Secondary Fish-Odor Syndrome Can be Acquired by Nitric Oxide-mediated Impairment of Flavin-containing Monoxygenase in Hepatitis B Virus-Infected Patients

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Primary fish-odor syndrome (FOS) is a genetic disorder caused by defective flavin-containing monoxygenase 3 gene (FMO3) with deficient N-oxidation of trimethylamine (TMA), causing trimethylaminuria (TMAU). By contrast, secondary FOS can be acquired by decreased FMO activities in patients with chronic liver diseases, but the underlying mechanisms are unknown. In the present study, we examined plasma NOx concentrations and viral DNA contents as well as in vivo FMO activities and their correlations in chronic viral hepatitis (CVH) patients. Plasma concentration of NOx was significantly increased by 2.1 fold (56.2 ± 26.5 vs. 26.6 ± 5.4 μM, p < 0.01), and it was positively correlated with plasma hepatitis B virus (HBV) DNA contents (r²=0.2835, p=0.0197). Furthermore, the elevated plasma NOx values were inversely and significantly correlated with in vivo FMO activities detected by ranitidine-challenged test (6.3% vs. 20.9%, r²=0.2109, p=0.0315). TMA N-oxidation activities determined in CVH patients without challenge test were also significantly low (73.6% vs. 95.7%, p < 0.05). In conclusion, those results suggested that secondary FOS could be acquired by the endogenously elevated NO in patients with CVH.

Key Words: Fish-odor syndrome, Flavin-containing monoxygenase, Trimethylamine, Chronic viral hepatitis, Nitric oxide, Ranitidine

INTRODUCTION

Fish-odor syndrome (FOS), also known as Trimethylaminuria (TMAU), is a metabolic disorder caused by an inherited defect in flavin-containing monoxygenase 3 gene (FMO3). Defective FMO3 causes reduced N-oxidation and increases the excretion of volatile and malodorous aliphatic tertiary amine, trimethylamine (TMA), in the urine, sweat, breath, and other bodily secretions (Al-Waiz et al., 1987a, b, c; Ayesh et al., 1993). FMOs are known to catalyze the oxygenation of N-, S-, and P-containing endogenous and exogenous compounds to their respective oxides (Ziegler, 1988; Cashman, 1995). Among five FMO isoforms known to be present in man, FMO3 is the predominant one found in adult liver. Physiologically, FMO3 is known to catalyze N-oxidation of TMA, a volatile gas with fishy smell produced from dietary choline and lecithin by intestinal microflora, to the non-volatile and hydropobic TMA N-oxide. Normal liver is endowed with high FMO capacity for an efficient first-pass oxidation of TMA to TMA N-oxide, which is then excreted in urine without any fishy smell (Al-Waiz et al., 1988; Dolphin et al., 1997). Failure of FMO3 to oxidize TMA has been known to produce FOS or TMAU. This was originally thought to be a rare disease, but apparently FOS is being found to occur much more commonly. Non-genetic FOS types are now recognized and classified as the transient secondary FOS caused by hormonal changes, infectious diseases, chronic liver diseases and drug treatments (Simenhoff et al., 1977; Marks et al., 1978; Dolphin et al., 1997; Mayatepek and Kohlmuller, 1998). Breath, urine and body odor of these patients are slightly focal and in some cases, smell like rotten fish (Mitchell et al., 1999).

Patients suffering from chronic viral hepatitis (CVH) are also known to have severely depressed hepatic drug oxidation capacity and this is associated with depressed cytochrome P-450 (CYP) (Khatserko et al., 1993) and FMO activities (Nakajima et al., 1998). The mechanisms underlying the loss of these enzyme activities are not well understood. In the liver of patients with CVH, hepatocellular damage, subsequent fibrosis and formation of porte-caval shunts, and reduced blood flow to reduced number of functional hepatocytes are observed frequently. Thus, cirrhosis and hepatocellular carcinoma (HCC) have been suggested to be responsible for the decreased metabolic clearance of odorous volatile compounds absorbed from the gut (Mitchell et al., 1999).

ABBREVIATIONS: FOS, fish-odor syndrome; TMAU, trimethylaminuria; FMO, flavin-containing monoxygenase; TMA, trimethylamine; TB, thiobenzamide; RA, ranitidine; NO, nitric oxide; CVH, chronic viral hepatitis.
Animal studies have shown that over-stimulation of immune system suppresses hepatic drug metabolism function. In support of this, treatment of experimental animals with an endotoxin such as lipopolysaccharide (LPS) leads to overproduction of nitric oxide (NO) by induction of inducible nitric oxide synthase (iNOS) in vivo and this over-produced NO has been suggested to suppress the hepatic drug metabolism functions by suppressing CYP (Khatzenko et al., 1993; Wink et al., 1998; Minamiyama et al., 1997) and FMO (Park et al., 1999). In addition to this NO-dependent suppression of CYP function, several studies have demonstrated that NO-independent mechanisms (probably cytokine-dependent pathways) are also involved in the suppression of CYP-mediated drug metabolism function (Monsouwer et al., 1996; Sewer & Morgan, 1997, 1998; Sewer et al., 1998; Morgan et al., 2001). In addition to CYP, NO-derived suppression of FMO1 expression, as demonstrated by our previous study (Park et al., 1999), suggested that overproduced NO in the liver of CVH patients may serve as an important modulator in suppressing the FMO activities required for oxidation of volatile nitrogenous compounds absorbed from the gut.

Thus, the present study was conducted to understand the possible mechanisms involved in the cause of secondary FOS in patients with HBV-positive chronic liver diseases by determining in vivo FMO activities, plasma load of HBV DNA contents, plasma NOx levels and their correlations.

METHODS

Chemicals

Ranitidine (RA) and trimethylamine (TMA) were purchased from Sigma Chemical Co. (St Louis, MO, USA). RA metabolites including demethyl RA and RA N-oxide, used as standards in high-performance liquid chromatography (HPLC) analysis, were kind gifts from Dr. Carol Jenkins of GlaxoWellcome (Research Triangle Park, NC, USA).

Patients and volunteers

Twenty-two patients diagnosed with chronic hepatitis B virus (HBV) infection admitted to the Inha University Hospital participated in this study. In some patients (n=8), CVH alone was demonstrated, but others had cirrhosis (n=7), or cirrhotic hepatocellular carcinomas (HCC, n=7) without gender grouping in small population size. These patients gave their written informed consent and the study protocol was approved by the Institutional Review Board at Inha University Hospital. Urine to determine RA (N-oxide following RA challenge test and TMA N-oxide without the RA challenge test) and plasma samples (to determine NOx concentration) were collected to determine the in vivo FMO activities in all 22 patients (58.6±10.2 yr, female: 6, male: 16, p > 0.05 compared with normal individuals) and in 12 normal healthy volunteers (49.8±16.7 yr, female: 5, male: 7). Especially, plasma HBV DNA contents were determined from 22 patients. All patients and volunteers were maintained on choline- or lecithin-restricted hospital diets containing minimal amounts of bean and meat products for one day before and during the day of urine collection. However, we did not control for the amount of food they consumed.

In vivo FMO activity with RA challenge test

Patients and volunteers were given a tablet of Zantac® (Glaxo-Wellcome Korea, Ltd) containing 168 mg ranitidine (RA) hydrochloride (equivalent of 150 mg RA base) and 8 h cumulative urines were collected as reported in our previous study (Kang et al., 2000). Volumes of urine were recorded and 10 ml aliquots were stored frozen for later HPLC analysis of RA metabolites. Briefly, aliquots (100 μl) of thawed urine samples were spiked with 200 nmol of quinine (internal standard) dissolved in 100 μl methanol. HPLC mobile phase used for initial 7 min isocratic elution consisted of 1 : 9 (v/v) mixture of acetonitrile and phosphate buffer (5 mM, pH 8.0, solution A). This was followed by 3 min linear gradient elution with solution B [7 : 3 (v/v) acetonitrile and phosphate buffer] and then continued for 8 min of isocratic elution with 100% solution B. The elute was screened between 280 nm and 340 nm for qualitative identification of metabolite peak positions and peaks were monitored at 320 nm for quantitative analysis. Individual in vivo FMO activity was estimated by calculating the ratio of urinary contents of ranitidine N-oxide (RANO) to RA.

In vivo FMO activity without RA challenge test

Cumulative 24 h urine samples were collected in containers, to which 10 ml of 6 M HCl had been added to trap the volatile trimethylamine base (TMA) from evaporation by converting it to a non-volatile and water-soluble form (TMA hydrochloride). Volumes of total urine collected from patients and volunteers were recorded and 20 ml aliquots were stored frozen for later analysis to determine the contents of TMA and its N-oxide, TMAO. Contents of TMA and TMAO were measured as previously described (Zhang et al., 1992). Briefly, the TMA hydrochloride contained in thawed urine sample was first made volatile by alkalizing and converting it back to TMA, and the content of free TMA present in gaseous phase of warmed vials was determined by using a gas-liquid chromatography system (head-space analysis, HP5890-series II). Subsequently, the content of total TMA (free TMA plus TMAO) was determined after chemically reducing the non-volatile TMAO present in urine samples back to the volatile TMA by adding titanian chloride and then quantifying this by head-space gas-liquid chromatography. The content of urinary TMAO was calculated by subtracting the free TMA value from the total TMA. The hourly production of free TMA, TMAO and total TMA present in cumulative 24 h urine samples was expressed in micromoles and the percentage of TMAO in total TMA was calculated to estimate the TMA N-oxidation capacity or the in vivo FMO activity.

Plasma concentration of NO metabolites (NOx)

Plasma was prepared from blood samples (10 ml) collected from each patient and normal volunteers and used to determine the amount of stable NO metabolites, nitrite and nitrate (NOx), using the Griess reaction method (Green et al., 1982). Briefly, an aliquot of plasma was mixed with equal volume of Griess reagent (1.0% sulfanilamide and 0.1% N-1-naphthylethylene diamine in 5% phosphoric acid). After 10 min incubation at room temperature, absorbance was measured at 540 nm using a PowerWave340 Elisa (Bio-Tek Ins. Inc. Winoskeh, VT).
**Plasma HBV DNA contents**

Plasma viral DNA contents were determined using Amplicor HBV monitor™ test kit (Roche, Branchburg, NJ, USA) according to the manufacturer's package insert instructions. Briefly, HBV was collected from plasma by polyethylene glycol 8000 precipitation, lysed by dilute NaOH, and neutralized. After PCR amplification, samples were transferred into a streptavidin-coated microwell plate, and the biotinylated samples were treated to bind the streptavidin. Following hybridization to the immobilized samples, DNP moiety of the probes was detected colorimetrically using an anti-DNP-alkaline phosphate conjugate and p-nitrophenylphosphate substrate. The HBV DNA contents were calculated from the ratio of optical density for the HBV-specific well.

**Statistical analysis**

Data are presented as means±S.D., but HBV DNA contents as median values. Significance of any differences was determined by simple unpaired student’s t tests (for mean values) or Mann-Whitney non-parametric U tests (for median values). Statistical significance was assumed at p values of <0.05. In addition, associations between plasma NOx concentrations and HBV viral DNA contents, and in vivo FMO activities were evaluated using the Pearson’s correlation.

**RESULTS**

**In vivo FMO activities**

As shown in Table 1, in vivo FMO activities estimated by percent ratio of RANO to RA (ranitidine challenge test) excreted in the urine of patients were significantly lower (8.3±0.7%) than those in normal healthy volunteers (20.0±2.7%, p<0.05, student’s t test). The in vivo RA N-oxidation (FMO) activity observed in patients with CVH only, without any indications of liver cirrhosis or HCC, was not significantly different from those obtained in patients with liver cirrhosis or with cirrhotic HCC (data not shown). In vivo FMO activities estimated by percentage of TMAO present in the urine of patients without the RA challenge test were significantly lower (average 73.6±2.4%) than those estimated in normal healthy volunteers (95.7±0.5%, p<0.05, Student’s t test) (Table 1).

**Concentration of nitric oxide (NO) metabolites**

As also shown in Table 1, plasma NOx concentration in all patients examined was 56.2±26.5 μM (range 21.0–110.3), and that of healthy volunteers was 26.6±5.4 mM (range 17.9–34.2). Plasma NOx level observed in patients with CVH with without indications of liver cirrhosis or HCC was not significantly different from those of patients with cirrhosis or HCC (data not shown).

**Plasma HBV DNA contents**

Plasma load of infected HBV was determined by employing the binding affinity of DNA probe which binds specifically to the HBV particles. Plasma HBV DNA contents of patients calculated from the ratio of optical density for

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<th>Healthy volunteers (n=12)</th>
<th>CVH patients (n=22)</th>
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<tbody>
<tr>
<td>RANO (μg/ml urine)</td>
<td>9.4±1.0</td>
<td>2.2±0.7*</td>
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<tr>
<td>RA (μg/ml urine)</td>
<td>45.6±7.6</td>
<td>27.3±2.3*</td>
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<td>RANO/R (%)</td>
<td>7.4±1.3</td>
<td>12.6±0.2</td>
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<tr>
<td>TMAO (μmol/l)</td>
<td>88.7±14.2</td>
<td>150.3±170.2*</td>
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<tr>
<td>TMAO (μmol/l)</td>
<td>58.0±111.0</td>
<td>11.2±873.0</td>
</tr>
<tr>
<td>TMAO (μmol/l)</td>
<td>366.4±252.1*</td>
<td>1213.3–2430.1</td>
</tr>
<tr>
<td>TMAO (μmol/l)</td>
<td>85.7±20.5</td>
<td>73.6±4.4*</td>
</tr>
<tr>
<td>Plasma NOx (μM)</td>
<td>26.6±5.4</td>
<td>56.2±26.5*</td>
</tr>
<tr>
<td>(17.9–34.2)</td>
<td>(21.0–110.3)</td>
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<tr>
<td>HBV DNA (log, copies/ml)</td>
<td>2.82</td>
<td>5.5±**</td>
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<td>(2.04–3.72)</td>
<td>(2.67–6.17)</td>
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NOTE: *% RANO excretion in urine was obtained by dividing the concentration of RANO by the RA concentrations present in 8 h cumulative urine samples following challenge with RA. **% TMAO excretion in urine was obtained by dividing the hourly TMAO excretion rate by the sum of TMAO and TMAO excretion rates. Numbers indicate the mean±SD values, and the numbers in parentheses indicate the ranges examined. Numbers indicate the median values. *p<0.05 & **p<0.0001: Significant difference from the values observed in healthy volunteers (Unpaired Student’s t tests & Mann-Whitney U-test, respectively).

**Correlations among plasma NOx concentrations, HBV DNA contents and in vivo FMO activities**

As shown in Fig. 1A, plasma level of NOx concentration were correlated positively with the level of plasma HBV DNA content, when tested by Pearson’s correlation (r²=0.2835, p=0.0107). Furthermore, the plasma NOx level was inversely correlated with the in vivo FMO activity determined by a challenge test with FMO-specific substrate ranitidine (r²=0.2109, p=0.0315) (Fig. 1B).

**DISCUSSION**

Results of this study indicated that mild fish-odor syndrome (FOS) can be induced by overproduction of nitric oxide (NO) in patients with chronic liver diseases caused by chronic viral hepatitis, infective cirrhosis and carcinoma. These suggested further that acquired secondary FOS may be caused by reduced FMO activity mediated by inflammatory NO.

In contrast to the primary FOS caused genetically by rare
Fig. 1. Correlation scatterplots between plasma NOx concentration and HBV DNA contents (A), and between in vivo FMO activity determined by RA N-oxidation capacity and plasma NOx concentration (B) in HBV-infected patients (Pearson’s correlation).

point mutations (Dolphin et al., 1997; Treacy et al., 1998) or common mutations (Zachocke et al., 1999; Park et al., 1999; 2002) in FMO3 gene, secondary FOS can occur in early childhood (Mayntopk & Kohlmuller, 1998), through an overload of dietary TMA precursors (Zhang et al., 1999), with age related decline of hepatic FMO activity (Chung et al., 2000; Koukouritaki et al., 2002) or with chronic liver diseases related decline of trimethylamine (TMA) oxidizing function (Mitchell et al., 1999). In particular, chronic infection of liver tissue by pathogens leads to profound reduction of hepatic function with altered expression of several genes and activities of enzymes encoded by these genes (Chisari & Ferrari, 1995). In individuals with acute or chronic liver diseases, as reported for CYPs, FMO activity involved in the oxidation of TMA to its major metabolite TMA N-oxide is suppressed (Khatzenko et al., 1993; Park et al., 1999).

Thus, in patients with chronic viral hepatitis, the ability of liver to metabolize systemically absorbed volatile substance TMA may fail and cause the disease-induced FOS through reduced or deficient FMO activity. In support of this, Mitchell et al. (1999) also suggested that in patients with severe hepatic disease excessive amounts of un-metabolized TMA might contribute to the cause of secondary FOS. Recently, Mitchell & Smith (2001) have reviewed several cases of FOS without suggesting the underlying mechanism involved in the suppression of hepatic FMO activity.

In the liver of animals infected by pathogens such as viruses, bacteria, or parasites, it is well-known that activities of hepatic enzymes metabolizing xenobiotics are significantly suppressed. In these pathogen infected conditions, inducible NO synthase (iNOS) is over-expressed in hepatocytes, Kupffer cells and stellate cells in liver tissues, and regional overproduction of NO has been demonstrated by several studies (Geller et al., 1983; Sälkowski et al., 1997; Muriel, 2000; Akaike & Maeda, 2001). Therefore, liver is a typical organ that is fully influenced by endogenous cytokines and that NO is overproduced acutely or chronically from both parenchymal and nonparenchymal cells. In particular, the overproduced NO is known to inhibit the activities of hepatic CYPs (Wink et al., 1993; Stadler et al., 1994) and cause subsequent depression of drug metabolism function (Khatzenko et al., 1993). There are much evidences supporting that endogenously overproduced NO inhibits not only the activities but also the expression levels of CYPs causing important changes in hepatic metabolism and disposition of several xenobiotics as well as administered drugs (Khatzenko et al., 1993; Wink et al., 1993; Stadler et al., 1994; Kim et al., 1995; Wink & Mitchell, 1996; Morgan et al., 2001).

In addition to NO, some cytokines are also known to suppress hepatic drug-metabolizing enzyme activities and expression of CYPs (Craig et al., 1990; Wright & Morgan, 1990; 1991; Stanley et al., 1991; Morgan, 1993; Sewer & Morgan, 1997; 1998; Sewer et al., 1998). Furthermore, inhibition of FMO gene expression by overproduced NO was also demonstrated in LPS-pretreated rat livers (Park et al., 1999). Transcription efficiency was suppressed and the overall enzymatic activity of hepatic FMO was reduced. Such reductions of FMO1 (the major form in rat liver) mRNA expressions and FMO activities were completely or partially protected by co-treatment with an iNOS-specific inhibitor, aminoguanidine (AG). These results indicated that NO inhibits FMO mRNA expression and suggested further that overproduced NO might cause the lower in vivo FMO activity in patients with liver diseases such as hepatitis, cirrhosis or cancer, and explain the secondary FOS. In this support, our recent study showed that the levels of FMO3 mRNA and protein expressed in liver tissues of cirrhotic and cancer, in which the iNOS expression was elevated, were markedly reduced (Ryu et al., 2004). However, it was not possible to determine whether or not NO causes the reduction of FMO3 mRNA expression in human subjects. Thus, in the present study, we have shown a significant inverse correlation between decreased in vivo FMO activity and elevated plasma NOx level and HBV DNA load in patients with CVH (Fig. 1). Our results support the observation of Majano et al. (1998) which showed that contents of hepatocellular iNOS mRNA and protein in the liver biopsy tissues of CVH patients were increased dramatically, although there was no mention for the involvement of NO in secondary FOS.

FMO activities (RA N-oxidation) determined in vitro using biopsy liver tissues obtained from CVH patients were much lower than that expected from the level of FMO3 protein found in the biopsy liver tissue (Ryu et al., 2004). Based on this observation, a direct suppressive effect of overproduced NO on FMO activities has been assumed as one of the primary causative mechanisms of secondary FOS.
in patients with chronic liver diseases. Supporting our assumptions, the lower-than-expected FMO activity was restored after treatment of liver microsomes with dithiothreitol (DTT), a sulfhydryl-reducing agent or ascorbate to reverse nitrosylation. This suggested that overproduced NO could induce or mediate a conformational change in FMO3 and reduce the FMO activity directly. In particular, such a post-translational modification of FMO3 can be caused by a direct binding of NO via the DTT or ascorbate reversible S-nitrosylation on the cysteine-thiol residue in FMO3, not to the FAD prosthetic group in FMO3 (Ryu et al., 2004). As suggested by Stamler et al. (1992), NO may cause S-nitrosylation of cysteine residue in the active site of FMO3 forming an S-nitrosothiol (RS-NO) and inhibit the enzyme activity. Therefore, the liver disease-induced secondary FOS may have been due, at least in part, to the S-nitrosylated FMO3 causing the lower-than-expected FMO activities.

In addition to the apparent inhibitory effect of enzymatically generated NO on FMO activities, exogenously added NO donors also inhibited the isolated rat liver microsomal FMO activities such that they were completely restored by DTT or ascorbate (Ryu et al., 2004). Although clear differences appear between the employed NO donors, the evidence presented here suggests that hepatic FMO proteins (FMO1 in rat and FMO3 in human) can be S-nitrosylated by the overproduced NO. Based on these current and previous results, we hypothesize that NO can inhibit pre-translational efficiency and also induce post-translational modifications of FMO3 leading to substantial reduction of in vivo FMO activities as detected by the urinary ratios of TMAO/TMA (TMAO) or RANO/RA (Table 1), resulting in acquired FOS in man.

In conclusion, our results indicate that overproduced NO in the liver can depress FMO3 activity and cause secondary FOS observed in vivo in patients with CVH. Overproduced NO resulting from induction of iNOS expression in liver tissues can decrease FMO3 expression indirectly and/or inhibit FMO activity directly via reversible S-nitrosylation. Both of these direct and indirect effects of NO may operate in concert to compromise the FMO3 activity. The overproduced NO is responsible, at least in part, for the acquired form of FOS observed in patients with CVH.

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